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<b>(54) Title:</b> DIAGNOSTIC TESTS MEASURING GELATINASE/INHIBITOR COMPLEXES FOR DETECTION OF AG- GRESSIVE AND METASTATIC CANCER  <b>(57) Abstract</b> <p>Diagnostic agents and methods for detecting the presence of metastatic activity in biological samples such as plasma are disclosed. The agent and method preferably immunologically detect matrix metalloproteinases in complexed form with endogenous inhibitors of MMP's. A kit for detecting the metalloproteinases is also disclosed.</p>		

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DIAGNOSTIC TESTS MEASURING GELATINASE/INHIBITOR COMPLEXES FOR DETECTION OF  
AGGRESSIVE AND METASTATIC CANCER

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BACKGROUND OF THE INVENTION

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The present invention relates to improvements in the diagnosis of metastatic disease. In particular, the invention relates to techniques for detecting the presence of proteolytic enzymes associated with metastatic disease.

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Although a cure for most forms of cancer remains elusive, early detection and treatment have historically provided the best prognosis. Considerable effort over the years has been directed to developing diagnostic tests which give an indication of the presence of metastatic disease at an early stage.

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Recently, a relationship between matrix metalloproteinases (MMP's) and metastasis has been suggested. MMP's are a family of closely related metal-dependent endopeptidases secreted by mesenchymal cells. For example, gelatinase A (MMP-2 or 72 kDa gelatinase/type IV collagenase) and gelatinase B (MMP-9 or 92 kDa gelatinase/type IV collagenase) have been identified as playing a major role in cancer invasion and metastasis. Stromelysin and PUMP (Putative Metalloproteinase), metalloproteinases with broader substrate specificity, are also thought to participate in the metastatic process. Three of these metalloproteinases have been identified in mammalian plasma.

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Metastasis is a complex sequence of events in which malignant cells detach and disseminate from a primary tumor. The malignant cells invade adjacent tissue, penetrate into blood and lymphatic vessels, circulate to distant sites and eventually attach to and penetrate tissues in distant organs, thereby proliferating the malignancy. An important part of the metastatic process is the degradation of extracellular basement membranes by various proteolytic enzymes.

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1 Basement membranes are collagen-containing connective  
tissues which form a tough continuous sheet and separate the  
various cell layers such as the epithelial, endothelial and  
parenchymal cells from interstitial connective tissue.

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Proteolytic enzymes associated with the metastatic  
process are found circulating in the form of activated  
enzyme (free enzyme), latent free enzyme and complexed with  
endogenous proteinase inhibitors. Only the active forms,  
10 however, digest the connective tissue substrates. The  
body's own natural defense mechanisms rapidly inactivate  
MMP's by complexing the enzymes with specific tissue  
inhibitors of metalloproteinases (TIMP's). Such inhibitors  
found in the tissues and circulating in the plasma include  
15 TIMP-1, TIMP-2 and also alpha-2 macroglobulin which is  
primarily in blood.

In spite of the relationship between elevated  
levels of certain destructive proteases and the presence of  
20 metastatic disease, it has been difficult to use the  
relationship to provide an accurate indication of metastatic  
disease. In the past, it has only been possible to detect  
free or activated forms of the metalloproteinases, leaving a  
substantial portion of the inactivated enzymes complexed  
25 with inhibitors undetected. Recently it has been shown,  
however, that latent gelatinase A forms complexes with TIMP-  
2 and latent gelatinase B forms complexes with TIMP-1.  
Thus, these gelatinases can exist outside the cell in  
complexed forms with TIMPs.

30

U.S. Patent No. 4,677,058 discloses purifying  
and detecting type IV collagenase antigens from malignant  
tumor cells. Similarly, U.S. Patent No. 4,808,528 discloses  
antibodies specific to type IV collagenase enzyme antigens.  
35 U.S. Patent No. 4,816,400, a division of the '058 patent,  
supra, discloses immunological determination of type IV

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1 collagenase antigens using polyclonal and monoclonal  
antibodies. None of these references, however, disclose  
detecting collagenase enzyme-inhibitor complexes associated  
with metastatic disease.

5

In spite of the investigation of the role of  
metalloproteinases in metastatic disease, the total amount  
of MMP's being released and thus the actual metastatic  
activity has not been detectable. Indeed, due to the  
10 usually rapid inactivation of activated MMP's by TIMP's,  
measuring only free, circulating MMP's would fail to  
indicate the presence of many underlying diseases.  
Determining whether or not the complexes formed between  
MMP's and TIMP's form a more reliable and a sensitive  
15 diagnostic tests has yet to be investigated.

It is therefore an object of the present invention to  
provide highly specific and reliable diagnostic agents and  
methods to determine the presence of metastatic disease  
20 based on measuring matrix metalloproteinase inhibitor  
complex levels.

Other and further objects and advantages of the  
present invention will become apparent to those skilled in  
25 the art from a consideration of the following description  
taken together with the accompanying figure.

#### SUMMARY OF THE INVENTION

30 The present invention includes diagnostic agents and  
methods useful in the detection of metastatic activity in  
biological samples such as human plasma. The invention  
includes a first substance capable of immunologically  
reacting with enzymes broadly described as matrix metallo-  
35 proteinases (MMP's). Such MMP's include gelatinase A 72 kDa  
type IV collagenase/gelatinase (MMP-2), gelatinase B 92 kDa

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- 1 type IV collagenase/gelatinase (MMP-9), stromelysin (MMP-3),  
putative metalloproteinase (PUMP), partial breakdown  
products of these proteins and combinations thereof. The  
invention further includes a second substance which is  
5 immunologically reactive with endogenous inhibitors of the  
tissue-degrading enzyme metalloproteinases or TIMP's.

The first immunologically-reactive substance is  
preferably a monoclonal antibody having specificity for one  
10 or more MMP enzymes or circulating breakdown products of  
MMPs. Examples of such antibodies include monoclonal murine  
anti-MMP-2, monoclonal murine anti-MMP-9 and monoclonal  
murine anti-MMP-3, such that set forth by Bergmann, et al.  
J. Clin. Chem. Clin. Biochem 27, 351-359 (1989) or Cell Tech  
15 Lmt. (Slough, England). Alternatively, antibodies such as  
rabbit polyclonal antibodies to native MMP's or peptide  
components such as peptide sequences of the native MMP's may  
be used.

20 The second immunologically responsive substance is  
preferably an antibody having specificity for endogenous  
inhibitors of MMP's, TIMP's or other associated proteins  
which bind to MMP's in plasma or tissue. For example,  
polyclonal rabbit anti-TIMP-1 or polyclonal anti-TIMP-2 as  
25 described by Carmichael, et al. Proc. Natl. Acad. Sci. USA,  
83:2407-2411 (1986) or the N.I.H. (Bethesda, MD) are  
examples of such substances. Alternatively, monoclonal  
antibodies to TIMP's may be selected.

30 The present invention also includes a method of  
detecting metastatic activity in a biological sample and  
a diagnostic kit. The method includes contacting a  
diagnostic agent, such as that set forth above, with the  
biologic sample and measuring the total amount of matrix  
35 metalloproteinases present and TIMP complexes uncovered to  
determine whether metastatic disease is present.

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1       The diagnostic agent, method and kit of the present  
invention can be included as part of various immunoassay  
techniques, particularly ELISA and most preferably sandwich-  
type ELISA assays. Alternative immunoassay techniques such  
5 as immunoblotting, immunofluorescent, radio-immunoassay,  
fluorescence detection and/or enzyme assay methods are also  
contemplated.

As a result of the present invention, significantly  
10 more accurate determinations of metastatic activity are  
obtained by detecting not only free metalloproteinases  
and breakdown products of MMP's, but also complexes of  
the enzymes formed with inhibitors. This is a dramatic  
improvement over methods which only detect free or activated  
15 MMP's and completely missed those enzymes complexed to the  
inhibitor molecules. In addition, the assays described  
herein provide independent verification of disease. Thus,  
the results provided can supplement other tests and provide  
additional data not obtained with other tests.

20

For a better understanding of the present invention,  
reference is made to the following description, taken  
together with the accompanying figure, and its scope will be  
pointed out in the appended claims.

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#### BRIEF DESCRIPTION OF THE DRAWING

Figure 1 graphically demonstrates the results of  
immunological assays carried out using sandwich-type ELISA  
30 techniques to detect metastatic activity in patients using  
the present invention.

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DETAILED DESCRIPTION OF THE INVENTION

The diagnostic agent and method of the present invention are based on the premise that high levels of certain metalloproteinases complexed to TIMP's in clinical samples strongly correlate with an underlying metastatic disease. Thus, while not necessarily indicating a particular type of cancer, the diagnostic agent and method of the present invention provide a means of indicating or affirming the presence of underlying metastatic disease in the biologic fluids of suspected patients.

Blood and/or plasma are the most common biologic fluids assayed for diagnostic tests. For purposes of the present invention, the term biologic fluids shall also include but not be limited to plasma, serum, tissue samples, aspirates, urine and tissue fluids. It is contemplated that the inventive diagnostic agent and method provide an indication of metastatic activity from any sample containing metalloproteinases in both free and complexed form, even in amounts as low as the nanogram per milliliter level.

The identification of metalloproteinases in clinical samples is preferably carried out using immunologic techniques. The immunologic techniques center around the use of specific antibody-antigen reactions which indicate a response to only specific antigens, in particular, those enzymes associated with metastatic disease. Within this genre of tissue-degrading enzymes are gelatinase A 72 kDa type IV collagenase/gelatinase (MMP-2), gelatinase B 92 kDa IV collagenase/gelatinase (MMP-9), stromelysin (MMP-3), putative metalloproteinase (PUMP's), and breakdown products of the proteinases and combinations thereof. For diagnostic purposes, it is of importance to note that the recognition of these antigenic substances is in both the free and complexed form.



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1 In order to achieve this result, the invention  
includes a first substance having immunospecificity to free  
metalloproteinases and complexed metalloproteinases. In a  
preferred embodiment, monoclonal antibodies are prepared to  
5 have the desired immunospecificity. For example, monoclonal  
antibodies may be obtained from hybridomas obtained from  
mice immunized by injection of 72 kDa procollagenase/type IV  
procollagenase purified from human fibroblasts. See  
Birkedal-Hansen, et al. Biochemistry 27, 6751-6758 (1988).  
10 It is essential that the antibody bind to the specific  
metalloproteinases when it is complexed to TIMP. Many  
antibodies currently used can recognize free metallo-  
proteinase, but they fail to bind to MMP in complexes with  
inhibitors as described in more detail below. A non-  
15 limiting list of suitable antibodies include murine  
monoclonal anti-human 72 kDa or 92 kDa type IV collagenase/  
gelatinase antibodies, or murine monoclonal antibodies to  
stromelysins. One method of producing these antibodies  
which recognize MMP's in complexes is to use MMP-TIMP  
20 complexes as the immunogen in mice or rabbits.

The inventive diagnostic agent also includes a second  
substance having particular specificity for the naturally-  
occurring tissue inhibitors of metallo-proteinases (TIMP's),  
25 including TIMP-1, TIMP-2, and alpha macroglobulin. These  
antibodies need to be reactive to TIMP's in complexes with  
MMP's.

Free TIMP-1 has a molecular weight of 28 kDa,  
30 but in complexes with MMP's, it is identified as  
approximately 95 kDa. Free TIMP-2 has a molecular weight of  
22 kDa, but complexed with MMP's, it is identified as  
multiple components with molecular weights between 23 kDa to  
150 kDa. Because gelatinase A and gelatinase B breakdown  
35 over time following activation or enzyme digestion, it is  
anticipated that lower molecular weight products of these

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- 1 MMPs may circulate in complexes with TIMP's. The molecular weights of TIMP's in plasma have been identified using immunoblotting techniques of non-reduced SDS-PAGE polyacrylamide gels.

5

- Preferably, antibodies which achieve the necessary binding to complexed TIMP are prepared specific to MMP-TIMP complexes found in the biologic sample. For example, polyclonal antibodies to human procollagenase/pro-type IV collagenase can be produced in rabbits by laboratory procedures known in the art. A good source for the immunogens is to isolate MMP-TIMP complexes from human plasma using gelatin Sepharose chromatography to bind these antigens and dimethyl sulfoxide to elute the complexed proteins from the solid phase. These antigens are mixed with Freund's Adjuvant to enhance antibody response and are injected subcutaneously in rabbits on 3-5 occasions over a period of 4-6 weeks. Further examples of such polyclonal antibodies include rabbit anti-TIMP-1, rabbit anti-TIMP-2 and anti-alpha-2-macroglobulin. Suitable antibodies are also available from commercial laboratories such as Cell Tech Ltd. (Slough, England).

- In an alternative embodiment, monoclonal antibodies are prepared with specificity to the TIMP complexes such as MAC-015 from Cell Tech Ltd. Combinations of antibodies are also contemplated. The monoclonal antibodies can also be prepared using laboratory techniques known to those of ordinary skill in the art, such as that provided by Cooksley, et al. MATRIX 10:285-291, 1990, or from commercial laboratory sources such as Cell Tech Ltd. The antibodies employed in this assay could also react to a new immunogen consisting of peptide components derived from a portion of the TIMP molecule and a portion of the MMP molecule.
- 35 Likewise, neoantigens could be produced as a result of the complexing of TIMP and MMP's, thus resulting in a unique

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- 1 antigen that would be diagnostic of the formation of  
complexes.

A preferred immunologic means of detecting  
5 metalloproteinases is the Enzyme-Linked Immunosorbent Assay  
(ELISA) method, and in particular the sandwich-type ELISA  
format. This assay method includes introducing a biologic  
sample between a capture layer of antibodies and a detection  
layer of antibodies.

10

In this regard, diagnostic well plates such as  
Immulon II 96 well microtiter plates available from  
Dynatech, Alexandria, VA are first coated with a capture  
antibodies directed to MMP's such as rabbit polyclonal  
15 antibodies to human 72 kDa gelatinase or 92 kDa  
progelatinase/type IV procollagenase. The capture antibody  
is introduced into the wells in amounts ranging from about  
10  $\mu$ l to about 200  $\mu$ l, with amounts of about 100  $\mu$ l being  
preferred. The capture antibody is preferably diluted in a  
20 suitable buffer such as 0.1 M NaHCO<sub>3</sub>, pH 9.0 to about 1:200  
concentration prior to introduction into the wells.

Binding of the capture antibody to the well is carried  
out over a period of from about 4 to about 24 hours and  
25 preferably about 18 hours after inoculation at temperatures  
ranging from about 0 to about 10°C and preferably about 4°C.  
Unbound antibody is thereafter removed by vigorously  
inverting the plates. The wells containing the bound  
antibody are next bathed with a bovine serum albumin/  
30 bicarbonate buffer to block excess binding sites on the  
wells. The bottom layer of the "sandwich" or capture layer  
is completed by washing the plate thoroughly with a buffer  
solution containing sodium phosphate, sodium chloride and  
Tween 20.

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1           The ELISA technique further includes introducing  
a biologic sample such as human plasma onto the capture  
antibody layer. The samples are preferably prepared by  
being diluted in an incubation-suitable buffer to about  
5 a 1:10 concentration. One such solution contains 50 mM  
sodium phosphate, 0.1 M sodium chloride, 0.02% Tween 20 and  
0.1% bovine serum albumin (BSA). The samples are placed in  
the well, incubated at a temperature ranging from about 25°C  
to about 37°C, and preferably at about 37°C for a time  
10 period of from about 1 hour to about 4 hours and preferably  
about one hour.

The final part of the ELISA "sandwich" is a detection  
layer containing an antibody specific to free TIMP-1 or  
15 TIMP-2 recognizing TIMP in complexes. The wells containing  
the capture antibody and sample are washed thoroughly before  
introducing a detection antibody into the well such as  
monoclonal murine anti-TIMP-1, anti-TIMP-2 antibodies or the  
like. The antibodies are preferably added after being  
20 diluted in an incubation buffer to about a concentration of  
about 1:4000.

The wells are incubated for about 1 hour and  
thereafter washed thoroughly and prepared using standard  
25 ELISA techniques known to the art, such as including the  
amplifying goat antibodies to mouse IgG and then alkaline-  
phosphatase conjugated to streptavidin. The wells are then  
washed and 100 microliters of substrate p-nitrophenyl  
phosphate in buffer is added to generate a color reaction  
30 which is read at  $A_{405}$ . The results of the assay are obtained  
using any suitable reading device such as that available  
from BioTek of Winooski, VT. In addition, the optimal  
concentration of capture antibodies such as polyclonal  
rabbit anti-gelatinase A, and detecting antibodies such as  
35 monoclonal murine anti-TIMP-1, is determined using  
checkerboard titration. In this procedure,

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- 1 the concentrations of different antibodies and antigens are  
varied by serial dilution of the reagents to determine the  
concentration of each reagent, giving the highest percentage  
of true positive results and the lowest percentage of false  
5 positive results.

While the present invention is not solely limited  
to ELISA immunologic techniques, the ELISA technique is  
particularly preferred since the method allows the artisan  
10 to detect concentrations of a particular substance, in this  
case MMP's, in nanogram per milliliter concentra-tions.  
Other suitable immunological techniques include radio-  
immunoassay (RIA), Western dot blot, dip stick, zymography  
as well as immunologic techniques known to those of ordinary  
15 skill in the art. For example, in certain techniques an  
absolute-type reading, i.e., a color change, indicates the  
presence of disease. In a preferred embodiment, a  
quantitative or numeric value indicating the level of  
enzyme-complex concentration or activity is provided thereby  
20 allowing comparison to a reference standard.

In another aspect of the present invention, a kit  
is provided for detecting the presence of metastatic disease  
in a biologic sample. The kit provides the inventive  
25 diagnostic agent as described herein and means for measuring  
the total amount of MMP complexed with circulating  
inhibitors thereof. In particular, the kit includes  
antibodies, preferably monoclonal or polyclonal antibodies  
against MMP's and a second type of antibodies, preferably  
30 monoclonal or polyclonal antibodies directed against TIMP's.  
The means for measuring can be any suitable means known to  
those of ordinary skill in the art such as antibody coated  
beads or other solid phase immobilized antibodies.  
Measuring may also be accomplished by Enzyme Linked  
35 Immunosorbent Assay (ELISA) radio-immunoassay, zymography  
and the like techniques.

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1        Other immunologic detection means can be readily  
adapted for use in connection with the diagnostic agent and  
method of the present invention. It is intended that all  
such alternative measuring and diagnostic means be included  
5 within the scope of the present invention.

With particular regard to the antibodies included  
herein, it will be appreciated by those of skill in the art  
that such antibodies, both monoclonal and polyclonal types  
10 are available from commercial sources such as Cell Tech Lmt.  
of Slough, England, or can be prepared using standard  
laboratory practices.

#### EXAMPLES

15        In the Examples set forth below, various aspects of  
the present invention are set forth to provide further  
appreciation of the invention. The Examples, however, are  
not meant in any way to restrict the effective scope of the  
20 invention.

#### EXAMPLE I

##### Sandwich-type ELISA Immunoassay For Detection of MMP-2 and 25 MMP-2/TIMP-2 Complexes In Human Plasma

In this Example, a preferred immunoassay is prepared.  
Initially, 96 well microtiter plates (Dynatech Immulon II,  
Alexandria, VA) were coated with 100µl of polyclonal rabbit  
30 anti-TIMP-2 (NIH, Bethesda, MD) diluted to a concentration  
of 1:200 in 0.1 M NaHCO<sub>3</sub>, pH 9.0 for 18 hours at 4°C. The  
unbound anti-TIMP-2 was removed and 200µl of 1% bovine serum  
albumin in bicarbonate buffer was added twice for 30 minutes  
at 27°C to block excess binding sites on the wells. The  
35 plate was washed 3 times with a washing buffer containing 50  
mM sodium phosphate, pH 7.2, 0.1 M NaCl, 0.05% Tween 20.

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1 Twenty-eight human plasma samples and six 100 $\mu$ l  
purified MMP-2/TIMP-2 complex standards were each diluted in  
an incubation buffer containing 50 mM sodium phosphate, pH  
7.2, 0.1M NaCl, 0.02% Tween 20, 0.1% bovine serum albumin,  
5 (BSA), and separately added to individual wells for 2 hours  
at 37°C. The wells were thereafter washed 3 times with  
washing buffer. Monoclonal murine anti-MMP-2 obtained from  
the University of Alabama, Birmingham, diluted 1:4000 in  
incubation buffer, was added in an amount of 100 $\mu$ l per well.  
10 The plates were incubated at 37°C for 1 hour.

The plates containing the ELISA sandwich were  
completed by being washed 3 times and adding 100 $\mu$ l of biotin  
labeled goat antibodies to mouse IgG, IgA, IgM (H&L chains)  
15 in a concentration of 1:1000 before being incubated at 37°C  
for 1 hour. The plates were again washed 3 times and  
alkaline-phosphatase conjugated to streptavidin (1:1000  
dilution, 100 $\mu$ l) was added to each well and the plates were  
incubated for 30 min. at 37°C. The biotin and streptavidin  
20 reagents are employed to amplify the signal from the  
detecting antibody reagent, thus permitting the detection of  
nanogram per ml concentrations of gelatinase-TIMP complexes.  
Alkaline phosphatase conjugated to streptavidin is the  
enzyme used to generate color from the p-nitrophenyl  
25 phosphate substrate.

The wells were washed 3 times with PBS-Tween  
phosphate-buffered saline with Tween detergent and 3 times  
with water. 100 $\mu$ l of substrate p-nitrophenyl phosphate  
30 (1mg/ml) in 0.1M glycine, 1mM MgCl<sub>2</sub>, 1mM ZnCl<sub>2</sub>, pH 10.4 was  
added and after 30-90 minutes at room temperature, the  
plates containing both the human plasma samples and the  
purified standards were read at A<sub>405</sub> in a Microplate  
Autoreader (BioTek EL 309. Winooski, Vt.). Quantification  
35 of MMP-2/TIMP-2 complexes was made by extrapolation from a  
log-log linear regression curve employing varying

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1 concentrations of purified MMP-2/TIMP-2 complex as standard.  
The results are set forth in the Table below and are  
graphically illustrated in Fig. 1.

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TABLE

	<u>DIAGNOSIS</u>	<u>STAGE</u>	<u>MMP-2</u> <u>(ng/ml)</u>	<u>MMP-2/</u> <u>TIMP-2*</u> <u>(units/ml)</u>	<u>MMP-9</u> <u>(ng/ml)</u>
5			Norm. Conc.= <u>0-828</u>	Norm. Conc.= <u>0-694</u>	Norm. Conc.= <u>0-31</u>
	<u>CONTROLS</u>				
10	A		610	288	0
	B		716	458	14
	C		677	423	17
	D		488	277	18
	E		615	271	8
	F		823	596	36
	G		496	556	1
	H		617	188	1
	I		1381	604	25
15	J		633	173	5
	K		683	434	2
	L		616	446	0
	<u>GI CANCER</u>				
20	A	3	480	1429	63
	B	3	774	377	4
	C	4	591	657	5
	D	4	312	406	22
	E	4	664	933	126
	F	4	658	596	4
	G	4	908	3277	45
	H	2	500	779	1
	<u>BREAST CANCER</u>				
25	A	4	622	804	1
	B	1	734	509	0
	C	3	1145	2428	51
	D	3	759	382	4
	E	4	786	2429	5
	F	4	1226	1278	71
	G	4	687	639	41
	H	3	734	593	-
30					

Key

35 \*Units/ml based on a reference standard purified from human plasma using gelatin sepharose chromatography followed by gel filtration chromatography.

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- 1 As shown in the above Table, the normal range was based  
on the mean  $\pm$  2 S.D. of 12 normal plasma samples collected  
in EDTA anticoagulant. Four out of eight GI cancer patient  
specimens and four out of eight breast cancer patient  
5 specimens (50%) had significantly increased levels of MMP-  
2/TIMP-2 complexes. These patients tended to have advanced  
cancer, indicating that this assay would be predictive of  
patients with metastatic cancer.
- 10 The MMP-2/TIMP-2 complex assay provided results that were  
independent from isolated measurements of either MMP-2 alone  
or MMP-9 alone. Some patients had elevations of MMP-2/TIMP-  
2 complexes and normal levels of MMP-2 or MMP-9, indicating  
15 enhancing the diagnostic utility of these assays. Results  
of MMP-2 measurements alone have not proven to be useful in  
the diagnosis of metastatic cancer. Furthermore, it is  
critical that the blood specimens obtained from patients be  
anticoagulated such as with EDTA since the levels of MMP's  
20 are falsely elevated in serum as a result of release of  
MMP's especially MMP-9 or gelatinase B from white blood  
cells during the clotting process.

#### EXAMPLES II-IX

- 25 In these Examples, sandwich ELISA assays were prepared in  
a manner similar that set forth in Example I, except that  
the capture and detection antibodies were varied so that  
different matrix metalloproteinases could be detected. The  
30 various combinations of immunoassay detection systems  
provide the clinician with a battery of diagnostic assays  
useful in the identification, diagnosis, treatment and  
determination of prognosis in patients with various types of  
cancer. The illustrative assays are set forth below.

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EXAMPLE	II	III	IV	V	VI	VII	VIII	IX
CAPTURE ANTIBODY TO DETECT TIMP SHOWN	TIMP-1	TIMP-1	TIMP-2	TIMP-1	TIMP-2	TIMP-2	TIMP-2	TIMP-1
DETECTION ANTIBODY TO DETECT MMP SHOWN	MMP-9	MMP-3	MMP-3	MMP-1	MMP-1	MMP-2	MMP-9	MMP-2

10

The above assays can be combined with other diagnostic methods such as (CEA) carcinoembryonic antigen measurements useful for colon cancer diagnosis and CA-125 (useful in ovarian cancer diagnosis) as a further or confirmational indicator of tumor growth and/or metastatic disease. It is to be understood that the above listed combinations are illustrative and in no way represent the complete range of possible assays which can be prepared in accordance with the present invention.

25

**EXAMPLE X**

In this Example, a sandwich ELISA format immunoassay was prepared in a manner such as that set forth in Example I. The assay in this Example, however, contained mouse monoclonal antibodies to human TIMP-1 MAC-015 antibody from Cell Tech Lmt. to capture TIMP-1/MMP-9 complexes and biotinylated monoclonal mouse antibodies to 92 kDa matrix metalloproteinases prepared according to the method set forth

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1 by Bergmann, et al. supra, the disclosure of which is  
incorporated by reference herein. The assay was used to  
compare the levels of these complexed antigens present in the  
plasma of patients with various types of cancer with those of  
5 non-cancerous controls. The cancer group was further defined  
as patients with gastrointestinal cancers (GI) and patients  
with female genitourinary tract (GU) cancers such as cancers  
of the ovary, cervix, vagina and uterus. The results are set  
forth in the Table below.

10

TABLE

15	GROUP n=sample size	MMP-9/ TIMP-1 COMPLEX INCREASE ONLY DETECTED	MMP-9 INCREASE ONLY DETECTED	MMP-9/ TIMP-1 COMPLEX AND MMP-9 INCREASE DETECTED	TOTAL NUMBER OF POSITIVES (%)
20	Controls n=49	1	1	1	3 (6%)
	GI cancer n=94	14	14	7	35 (37%)
25	GU cancer n=23	8	3	4	15 (65%)

Referring now to the Table, it can be clearly seen that there  
are significant analytical advantages in measuring the levels  
30 of MMP complexes in addition to free MMP's to detect or  
confirm metastatic disease.

The above data dramatically illustrate this point for  
patients with GU cancer. Consider that by measuring only  
35 MMP-9, the clinician was able to confirm the presence of  
metastatic disease in only 3 of 23 (13%) known metastatic

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1 plasma samples. However, when the clinician also assays  
complexes of the enzyme and combinations of free enzymes and  
complexes, the summation of all assays provides a much more  
accurate diagnostic indicator. Moreover, the data obtained  
5 from measuring TIMP/MMP complexes is independent from that  
obtained by measuring MMP-9 assays alone. The present  
invention, therefore, provides supplemental information  
regarding metastatic disease. Patients with other types  
of cancer may have elevations of different types of MMP  
10 complexes thus necessitating the performance of a battery of  
different tests such as that set forth in Examples II-IX to  
optimize the diagnostic potential of detecting aggressive  
cancer at an earlier stage.

15 The above Example provides further evidence of a strong  
link between the invasive/metastatic process and the  
increased levels of metalloproteinases, both free and with  
inhibitor complexes in human plasma. Moreover, there is  
also a positive correlation between highly metastatic cancer  
20 cell lines (phenotypes) and increased secretion of certain  
enzyme complexes. The practitioner, therefore, can custom  
tailor the assay, for example, by substituting capture and  
detection antibodies to measure specific antigens. It is  
contemplated that assays for specific metastatic diseases  
25 such as breast and/or gastrointestinal cancers could be  
developed based on the results obtained from subjecting a  
biologic sample to a battery of specific antibodies.

While there have been described what are presently  
30 believed to be the preferred embodiments of the invention,  
those skilled in the art will realize that changes and  
modifications may be made thereto without the parting from  
the spirit of the invention, and it is intended to claim  
all such changes and modifications as fall within the true  
35 scope of the invention.

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WHAT IS CLAIMED IS:

1. A diagnostic agent for detecting the presence of metastatic activity in a biologic sample, comprising:
  - a) a first immunologically responsive substance capable of reacting with matrix metalloproteinases; and
  - b) a second substance capable of reacting with endogenous inhibitors of matrix metalloproteinases.
2. The diagnostic agent of Claim 1, wherein said matrix metalloproteinases are selected from the group consisting of 72 kDa type IV collagenase/gelatinase A (MMP-2), 92 kDa type IV collagenase/gelatinase B (MMP-9), stromelysin (MMP-3), putative metalloproteinase (PUMP) and mixtures thereof.
3. The diagnostic agent of Claim 1, wherein said first responsive substance is capable of reacting with breakdown products of matrix metalloproteinases.
4. The diagnostic agent of Claim 1, wherein said first responsive substance is selected from the group consisting of monoclonal antibodies, polyclonal antibodies and mixtures thereof.
5. The diagnostic agent of Claim 4, wherein said first responsive substance is selected from the group consisting of murine monoclonal anti-human 72 kDa type IV gelatinase antibodies, 92 kDa type IV gelatinase antibodies and anti-human stromelysin and anti-human PUMP and mixtures thereof.
5. The diagnostic agent of Claim 1, wherein said inhibitors of matrix metalloproteinases are selected from the group consisting of tissue inhibitor of metalloproteinase (TIMP-1), tissue inhibitor of metalloproteinase (TIMP-2) alpha-2 macroglobulin and mixtures thereof.

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7. The diagnostic agent of Claim 1, wherein said second substance is a polyclonal antibody select from the group consisting of rabbit anti-TIMP-1, rabbit anti-TIMP-2 and anti-alpha-2 macroglobulin.

8. The diagnostic agent of Claim 1, wherein said second substance is a monoclonal antibody to one of TIMP-1, TIMP-2 and mixtures thereof.

9. The diagnostic agent of Claim 5, wherein said monoclonal antibody is selected from the group consisting of antibodies to fragments of gelatinase A or gelatinase B.

10. The diagnostic agent of Claim 1, wherein said first substance is present in an amount of from about 0.01ng/ml to about 2000 ng/ml by weight of said biological sample and said second substance is present in an amount of from about 0.01  
5 ng/ml to about 2000 ng/ml by weight of said biological sample.

11. The diagnostic agent of Claim 10, wherein said first substance is present in an amount of from about 0.01 ng/ml to about 2000 ng/ml by weight of said biological sample and said second substance is present in an amount of from about 0.01  
5 ng/ml to about 2000 ng/ml by weight of said biological sample.

12. A method for detecting the presence of metastatic activity in a biologic sample, comprising:

contacting said biological sample with a diagnostic agent capable of indicating the presence of matrix metalloproteinases (MMP's) and tissue inhibitors of MMPs.  
5

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13. The method of Claim 12, wherein said matrix metalloproteinases are selected from the group consisting of 72 kDa type IV collagenase/gelatinase A (MMP-2), 92 kDa type IV collagenase/gelatinase B (MMP-9) and stromelysin (MMP-3) and putative metalloproteinase (PUMP).

14. The method of Claim 12, wherein said first responsive substance is capable of reacting with breakdown products of matrix metalloproteinases.

15. The method of Claim 12, wherein said first responsive substance is selected from the group consisting of monoclonal antibodies, polyclonal antibodies and mixtures thereof.

16. The method of Claim 15, wherein said first responsive substance is selected from the group consisting of murine monoclonal anti-human 72 kDa type IV gelatinase antibodies, 92 kDa type IV gelatinase antibodies and anti-human stromelysin antibodies and anti-human PUMP antibodies.

17. The method of Claim 12, wherein said inhibitors of matrix metalloproteinases are selected from the group consisting of tissue inhibitor of metalloproteinase (TIMP-1), tissue inhibitor of metalloproteinase (TIMP-2) and alpha 2 macroglobulin.

18. The method of Claim 17, wherein said second substance is a polyclonal antibody selected from the group consisting of rabbit anti-TIMP-1, rabbit anti-TIMP-2 and anti-alpha-2 macroglobulin.

19. The method of Claim 12, wherein said second substance is a monoclonal antibody to one of TIMP-1, TIMP-2 and mixtures thereof.



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20. The method of Claim 16, wherein said monoclonal antibody is selected from the group consisting of antibodies to fragments of gelatinase A or gelatinase B.

21. A kit for detecting the presence of metastatic disease in a biologic sample, comprising:

a) a first means for detecting the presence of matrix metalloproteinases;

5           b) a second means for detecting the presence of endogenous inhibitors of matrix metalloproteinases thereof; and

10           c) means for measuring the amount of matrix metalloproteinases detected by said first and said second means.

22. The kit of Claim 21, wherein said first means is an antibody against said matrix metalloproteinases and wherein said second means is an antibody against said tissue inhibitors of matrix metalloproteinases.

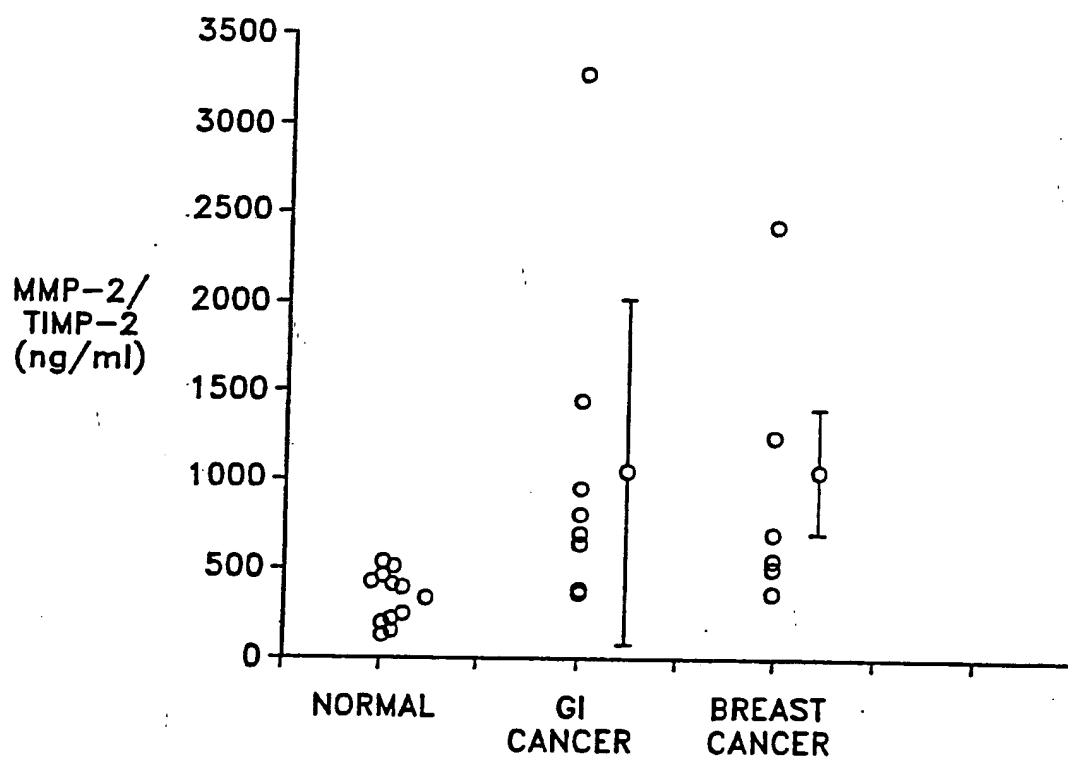
23. The kit of Claim 21, wherein said means is a monoclonal antibody and said second means is a monoclonal or polyclonal antibody.

24. The kit of Claim 21, wherein said means for measuring is an immunoassay.

25. The kit of Claim 24, wherein said means for measuring is an enzyme linked immunosorbent assay.

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FIG-1



SUBSTITUTE SHEET

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/02967

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : G01N 33/574; C07K 15/28; C12Q 1/37

US CL : 435/7.23, 7.92, 7.94, 23; 530/387.1, 388.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.23, 7.92, 7.94, 23, 810; 436/518; 530/387.1, 388.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<u>X</u> Y	MATRIX, Volume 10, issued 1990, S. Cooksley et al., "Immunoassays for the Detection of Human Collagenase, Stromelysin, Tissue Inhibitor of Metalloproteinases (TIMP) and Enzyme-Inhibitor Complexes," pages 285-291, especially page 285, column 1, first paragraph; page 286, column 1 and column 2, first paragraph.	<u>1, 4, 12, 15</u> 2, 3, 5-11, 13, 14, 16-25



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search

23 JUNE 1993

Date of mailing of the international search report

30 JUN 1993

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/02967

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Biol. Chem, Volume 264, number 29, issued 1989, W.G. Stetler-Stevenson et al., "Tissue Inhibitor of Metalloproteinase (TIMP-2)," especially page 17347, column 1, first paragraph and column 2; page 17377, column 2.	2, 3, 5-11, 13, 14, 16-25

Form PCT/ISA/210 (continuation of second sheet)(July 1992)\*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/02967

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

Dialog: Biosis, CAB Abstracts, Medline, Embase, Cancerlit, Derwent  
search terms: metasta?, plasma, metalloproteinase, MMP, collagenase, gelatinase, stromelysin, PUMP, macroglobulin,  
tissue(w)inhibitor, metallophthalein

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